

Original Research Article

GAD65 antibody prevalence and association with c-peptide, HLA class II alleles in Beninese patients with type 1 diabetes

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ABSTRACT

Background: Antibodies to glutamic acid decarboxylase and particularly their isoforms in 65 kDa are one of markers for the diagnosis of the type 1 diabetes (T1D). The aim of this study is to assess the prevalence of GAD65 antibodies (GAD65Ab) and investigate the association of GAD65Ab with C-peptide values, HLA Class II alleles genotyping. The diagnosis of T1D was set up according to American Diabetes Association criteria.

Methods: Radioimmunoassay was used to determine the GAD65Ab and C-peptide values. Class II HLA genotyping was performed in 51 patients with T1D and 51 healthy unrelated as control by using the PCR-SSP method. The sensitivity and specificity of the tests were calculated by standard formula.

Results: Result revealed that GAD65Ab were present in 74.5% (38/51) of the patients with T1D. There was no significant difference between the positivity or the negativity of GAD65Ab and gender, onset and duration of diabetes, frequencies of HLA-DR4, HLA-DR3-DR4, HLA-DQB1*0201. However, GAD65Ab values are linked to C-peptide concentration ($\chi^2 = 15.73$, $P = 0.0001$), the presence of HLA-DR3 ($\chi^2 = 9.75$, $P = 0.002$), HLA-DQA1*0501 ($\chi^2 = 4.09$, $P = 0.043$) alleles. The GAD65Ab test sensitivity and specificity were 74.5% and 94.1%, respectively. The C-peptide test showed a sensitivity around 82.4 % and 86.3 % for the specificity.

Conclusions: GAD65Ab showed to be a valuable early predictive marker and is associated with the risk to develop of T1D.

Keywords: C-peptide, GAD65, HLA Class II

INTRODUCTION

The type 1 diabetes (T1D) is a chronic autoimmune disease due to β -cell destruction, usually leading to absolute insulin deficiency.¹ This process takes place slowly during several years. During this reaction, auto-antibodies directed against some pancreatic antigens are produced. These antigens do not have in fact any

pathogenic role, but there are reliable to pathological auto-immune process markers. Further to the destruction of β cells, the production of endogenous insulin is suspended, hyperglycemia and ketoacidosis are then engendered, causing diverse complications.²

Nowadays there are more than 10 antibodies identified as markers of auto-immunity in the T1D, among which 04

types are dominantly used in clinical practice which are anti-ICA (islet cell antibody), anti-IA-2 (tyrosine phosphatase-like insulinoma antigen 2), anti-IAA (insulin autoantibodies), and anti-GAD (glutamic acid decarboxylase). Although, the ICA and the IAA are considered as T1D predictive markers, their specificity and sensitivity are too low.³

Anti-GAD are good markers to detect people with risk to develop T1D, especially in association with anti-ICA and because of their high sensitivity (90 %).⁴ In contrast to ICA antibodies, anti-GAD can still be detected in blood many years after the clinical disease onset.^{5,6} Two isoforms of GAD are identified with molecular weights of 65,000 (GAD65) and 67,000 (GAD67).⁷ The smaller-molecular weight antigen, GAD65, is the predominant form which is found in human pancreas. GAD65 antibodies might be predictive and diagnosis attractive markers according to longitudinal studies which showed little change on GAD65 antibodies levels before and after the onset of diabetes.⁸⁻¹⁰ There were only few studies concerning how HLA alleles relate to GAD autoimmunity.^{11,12} In addition, some Asian population have shown rather low frequencies (5-50%) of GAD65 and ICA in T1D when compared with Caucasian recent onset T1D patients (63-84%).¹³⁻¹⁸ GAD65Ab can be measured by radioimmunoassay.¹⁹ Using this method, Akamine and co-workers, found higher prevalence of GAD65Ab in Japanese type 1 diabetes patients.²⁰ Nevertheless in Benin any data was available, about the prevalence of GAD65 in patients with T1D.

The purpose of this study was: (1) to determine the prevalence of GAD65Ab; (2) to report any association of GAD65Ab with fasting C-peptide levels and HLA class II alleles in Beninese patients with T1D.

METHODS

Study sample

We investigated 51 patients with T1D who consulted in "insulin center" of Cotonou (a reference center for the diagnosis and the management of diabetics in Benin) from November 2012 to April 2015. A total of 51 unrelated healthy individuals without diabetes or a family history of diabetes in the general population were used as control group. They were paired to patients with T1D according to their age-, sex-, ethnic-, residence area for the study of the sensitivity and specificity.

The study was approved by the University of Abomey-Calavi Institutional Ethics Committee (IEC). All participants provided written informed consent.

Case ascertainment

Patients with T1D were diagnosed by a physician based on these following criteria: a fasting glycaemia ≥ 1.26 g/dl, an unexplained weight loss, signs of hyperglycemia

(polyuria, polydipsia, polyphagia, and asthenia) and an absolute insulin-dependence. These criteria were defined according to the recommendations of the American diabetes association (ADA).²¹

Participant eligibility

All participants must be originated from Benin. The patients with T1D must be diagnosed before 30 years old. Participant suffering from a serious illness in acute phase or under a drug which could interfere with the insulin sensitivity (corticoids, growth hormone, etc.) were excluded.

Participant characteristics

During the period of November 2012 to January 2016, a total of 102 participants were recruited (23 males and 28 females; sex-ratio = 1.21). Of these, 51 were T1D diagnosed and 51 without T1D (Table 1).

Table 1: Ages of study participants.

Age range	Patients with T1D	Controls
	n (%)	n (%)
< 5	04 (7.8)	04 (7.8)
5-15	39 (76.5)	39 (76.5)
>15	08 (15.7)	08 (15.7)

Radioimmunoassay technique

Each participant provided 5 ml of peripheral blood sample in anticoagulant free tube which was centrifuged afterwards.

The used procedure used was provided with the radioimmunoassay kit for anti-GAD65 (anti-GAD RIA; Ref: DEDAD100) and fasting C-peptide (IRMA CPEP; RÉF: 2179666) according to the manufacturer recommendations.²² Briefly two monoclonal antibodies were prepared against sterically remote sites on the GAD65 antibodies. The first one is coated on the solid phase (coated tube), the second one radiolabeled with iodine 125, is used as a tracer. Anti-GAD present in the calibrators or the samples to be tested are "sandwiched" between the two antibodies. Following the formation of the coated antibody/antigen/iodinated antibody sandwich, the unbound tracer is easily removed by a washing step. The radioactivity bound to the tube is proportional to the concentration of anti-GAD present in the sample. The same principle was used for the C-peptide dosage.

HLA Class II genotyping

Each participant was invited to provide a 5 ml of peripheral blood sample in EDTA tube, which was frozen at -20°C at the cytogenetic and medical genetic laboratory of the faculty of health sciences, university of Abomey-Calavi. The DNA extraction was performed

according to the phenol-chloroform standard technique. The DNA quality and its concentrations were determined by thermo scientific evolution 60S UV-visible spectrophotometer.

HLA Class II genotyping was performed with PCR-based on the sequence-specific primers (PCR-SSP) in a 20 µL mixture of 6.5 µL of DNA (100 ng/µL), 0.8 µL of MgCl₂ (2mM), 2µL of PCR buffer 1×, 2 µL of each deoxynucleotide triphosphate (200 µM), 4 µL of forward primers (20 µM), 4 µL of reverse primers (20 µM), and

0.7µL of polymerase Taq 0.175 U (invitrogen).²³ The process of DNA amplification is described as follow: The program of amplification contains an initial denaturation of 2 min in 94°C, 32 cycles of amplification (every cycle consists of a denaturation of 30s in 94 °C, a hybridization of primers during 30s in 63°C and an extension of 30s in 72°C) and a final extension of 10 min in 72°C. The products of amplification were separated on a 2%-agarose gel. The sequences of primers used for the amplification of the genes are presented in the Table 2.

Table 2: Sequences of the couples of primers used for the amplification of the genes.

Primers	Sequences
HLA-DR3	Fw: 5' CACGTTTCTTGGAGTAC 3'
	Rv: 5' CGTAGTTGTGTCTGCAGTAGT 3'
HLA-DR4	Fw: 5' CAGGTTAAACATGAGTGTCAATTTCTTAAAC 3'
	Rv: 5' GCTGTCTGAAGCGCACGTA CTCTTGGTG 3'
HLA-DQA1*05:01	Fw: 5' ACGTCCCTCTGGCCAGTA 3'
	Rv: 5' AGTTGGAGCGTTTAATCAGAC 3'
HLA-DQB1*02:01	Fw: 5' GTGCGTCTTGTGAGCAGAAG 3'
	Rv: 5' GCAAGGTCGTGCGGAGCT 3'
HLA-DQB1*06:02	Fw: 5' CGTGCGTCTTGTGACCAGAT 3'
	Rv: 5' GCTGTTCCAGTACTCGGCAT 3'
HLA-DQB1*03:02/3	Fw: 5' GACGGAGCGCGTGCGTTA3'
	Rv: 5' AGTACTCGGCGTCAGGCG3'
HLA-DQB1*03:03	Fw: 5' GACGGAGCGCGTGCGTTA3'
	Rv: 5' CTGTTCCAGTACTCGGCGT3'

Fw for forward; Rv for reverse.

Statistical analysis

Data are presented as means and standard errors. The significance of difference between observations was assessed using the χ₂-test or Fisher’s exact probability test where appropriate. A P value of 0.05 or less was deemed significant. The indices of sensitivity and the specificity values were calculated as follows:

$$\text{Sensitivity} = [a / (a+c)] \times 100$$

$$\text{Specificity} = [d / (b+d)] \times 100$$

Where a is the number of true positive samples, b is the number of false positive samples, c is the number of false negative samples and d is the number of true negative samples.²⁴

RESULTS

To determine whether GAD65Ab is associated to T1D in Beninese patients, we measured the prevalence of GAD65Ab in 51 patients with T1D. We found that the

prevalence of GAD65Ab was 74.5% (38/51) in patients with T1D.

The sensitivity and specificity for radioimmunoassay technique have shown 74.5% and 94.1% for GAD65Ab, and 82.4% and 86.3% for C-peptide respectively (Table 3).

The Link between the presence or not of GAD65Ab and studied variables were assessed. GAD65Ab values were found to be linked to C-peptide concentrations, HLA-DR3 and HLA-DQA1*0501 with P ≤ 0.05.

In contrast, GAD65Ab positivity is not linked to gender, duration of disease, HLA-DR4, HLA-DR3-DR4 and HLA-DQB1*0201 with P > 0.05 (Table 4).

The results showed that GAD65Ab remain detectable for a long time in the blood after a diagnosis of type 1 diabetes.

Figure 1 relates the percentage of the presence of GAD65 Ab vary according to the duration of T1D. From 0-5 years, the presence of GAD revolves around 68%. GAD65 Ab was present in all our patients who are

diagnosed 6 years after the development of T1D. From 7- around to 64% (Figure 1). 21 years after diagnosis, the presence of GAD decreases

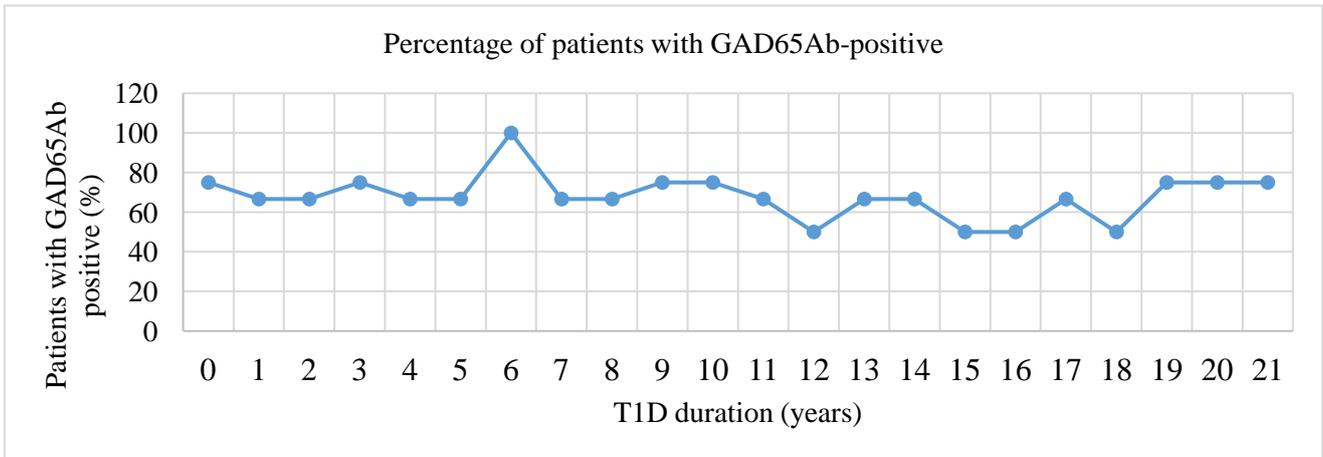


Figure 1: Positivity of GAD65Ab according to T1D duration in patients with T1D.

Table 3: Sensitivity and specificity measure of GAD65Ab and C-peptide in participants of study.

	Patients with T1D, n= 51	Controls, n= 51	Sensitivity (%)	Specificity (%)
GAD65Ab positive	38 (a)	03 (b)	74.5	94.1
GAD65Ab negative	13 (c)	48 (d)		
C-peptide low	42 (a)	07 (b)	82.4	86.3
C-peptide regular	09 (c)	44 (d)		

Prevalence of GAD65Ab is 74.5% and C-peptide is 82.4 % in patients with T1D.

Table 4: Link between GAD65Ab positivity and variables studied in Beninese patients with T1D.

Variables	GAD65Ab+ (N=38)	GAD65Ab- (N=13)	χ^2
Male/ Female	15/23	08/05	1.90
Onset/ after diagnosis	05/33	03/10	0.72
C-peptide +/-	36/02	06/07	15.73*
DR3+/-	35/03	07/06	9.75†
DR4+/-	12/26	03/10	0.003
DR3/DR4+/-	12/26	01/12	2.91
DQB1*0201+/-	19/19	07/06	0.06
DQA1*0501+/-	29/09	06/07	4.09††

*p < 0.001, †p < 0.01, ††0.01 < p < 0.05, p-values of the other values of χ^2 are > 0.05.

DISCUSSION

The presence of GAD65Ab is specific to T1D.⁴ Present study has provided the evidence that GAD65Ab is a valuable predictive marker of T1D before the onset of clinical symptoms. We have determined the prevalence and persistence of GAD65Ab using radioimmunoassay technique in patients with T1D. The sensitivity and specificity were evaluated by assessing GAD65Ab and C-peptide. GAD65Ab prevalence was estimated at 74.5 %. The presence of GAD65Ab reflects the auto-immune character of the T1D.

The prevalence found in the type 1 diabetics was situated in the interval of those described by other authors (52-75 %) like Rodacki and co-workers.²⁵ The Figure 1 shows that GAD65Ab remains detectable in blood long time after the diagnosis of type 1 diabetes with a peak of 100% observed after 6 years of T1D. Therefore, the investigation of GAD65Ab is very useful to identify the latent autoimmune diabetes cases in adult (LADA).²⁶

In the patients with type 2 diabetes, the presence of GAD65Ab indicates that an autoimmune destruction is in progress and that the diabetes evolves probably towards

the latent shape of the T1D (LADA) and thus towards an insulin-dependence.²⁶

According to Muller and co-workers (2010), at the T1D onset, prevalence of the GAD65Ab is from 70 to 80 % and often persists after the diagnosis.²⁷ Indeed, their positivity in an obvious clinical context of ketoacidosis, cardinal syndrome in a young subject of normal weight allows to assert the diagnosis.

Elevated levels of GAD65Ab were reported in 70 % of the cases of Stiff Man syndrome, a scarcity neurological disease which affects mainly women.²⁸ This may explain the presence of false positives that we have found in this study. This situation may also be explained by the existence of prediabetes.

However, it would be interesting to investigate these false positives. The deficiency of C-peptide secretion observed in 82.4 % of patients with T1D confirms that there are no insulin secretion and allow, consequently, their classification in the T1D. This absence of secretion is due to the destruction of the pancreatic islet (beta) cells producing insulin. In fact, C-peptide and insulin are secreted in equimolar amounts. Therefore, quantifying the C-peptide allows to extrapolate that of insulin since the half-life time of insulin is very short. This is the reason of the clinical interest of the serum assay of C-peptide.

Various techniques allow to measure GAD65Ab such as the radio-immunoassay, the fluorescent assay and ELISA. Bingley and co-workers (2003), studied the sensitivity and the specificity of these three techniques. Their result showed that the radioimmunoassay presents the best values of sensitivity (88 %) and of specificity (100%) when compared to the fluorescent assay (sensitivity 60 %; specificity 96 %) and to Elisa (sensitivity 72 %; specificity 92%).⁴

The study of the sensitivity and the specificity of the tests used in the present study showed that the sensitivity of GAD65Ab by radioimmunoassay was 74.5 % and 94.3 % for the specificity. The test of the C-peptide revealed a sensitivity of 82.4 % and a specificity of 86.3 %. This explains the existence of a correlation between T1D and GAD / C-peptide. Indeed, a diagnostic test with a good sensitivity leads to a positive result in almost all the patients with T1D. Thus, it is it could be used as a useful tool of diagnosis.

Moreover, a test with a good specificity, involves a negative result in almost all the control patients. It may be useful to measure GAD65Ab when it is difficult to distinguish the type 1 diabetes from the type 2 diabetes or when the probability of progress of the type 2 to the type 1 is raised.²⁹ One of the interests of this assay is to predict the appearance of the insulin-dependence and then the earlier insulin therapy.

Present study also showed that there is no link between the presence of GAD65Ab and the moment of the diagnosis of diabetes (Onset / after diagnosis). Our results, although no consistent with some studies, support the viewpoint that the GAD65Ab, declines slowly and persists in the blood for years.³⁰⁻³⁴

Study results showed also no link between GAD65 prevalence and gender. These results were like those of Chuang and co-workers and Chan and co-workers whereas Thai and al. reported GAD65 more frequent in females than males.^{30,33,35} Concerning the association between GAD65Ab and HLA alleles, this study showed a link between presence of GAD65Ab and HLA-DR3, HLA-DQA1*05:01. This relation with HLA-DR3 sustained the observations of Serjeant son and co-workers.³⁶ Likewise, Hagopian and co-workers have previously shown the existing relation between GAD65Ab and HLA-DQA1*05:01.³⁷

CONCLUSION

In conclusion, it exists a link between the presence of GAD65Ab and C-peptide, HLA-DR3 and HLA-DQA1*05:01. But further investigations are required to understand the effect of the presence of HLA genes on GAD65 autoimmunity in patients with T1D.

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